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THE MEMBRANE ATPase OF *ESCHERICHIA COLI*

I. ION DEPENDENCE AND ATP–ADP EXCHANGE REACTION

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SUMMARY

The properties of the membrane-bound ATPase (EC 3.6.1.3) of *Escherichia coli* have been reexamined using membranes obtained by mechanical disruption of exponentially growing cells.

The activity exhibited an absolute requirement for Mg^{2+} in the neutral pH range, while Ca^{2+} was found able to activate ATPase at more alkaline pH. Optimal activity was observed at pH 7.5, with a Mg/ATP ratio of 0.5.

ADP was found to inhibit ATP hydrolysis and to transform the Michaelian ATP concentration dependence with a K_m of 0.5 mM into a sigmoid curve with increasing K_m and decreasing V.

In contrast ADP activated an ATP–ADP exchange process and this shift from hydrolysis to exchange was stimulated by high Mg^{2+} and by orthophosphate.

All nucleoside triphosphates tested interfered with ATP hydrolysis, all could be hydrolyzed and could donate their terminal phosphate group to ADP. The relative efficiencies of nucleoside triphosphates in these three processes varied in parallel with minor discrepancies.

ATP hydrolysis was inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD) Dio 9, NaN_3 and pyrophosphate, the first two being ineffective against ATP–ADP exchange, the third being stimulatory and the last inhibitory.

ATP hydrolysis and ATP–ADP exchange are tentatively attributed to the terminal enzyme of oxidative phosphorylation.

INTRODUCTION

ATP is the predominant high energy metabolite in all organisms. Bacteria such as *Escherichia coli* form no exception. They have no other quantitatively important form of energy storage. The main trend of evolution in this species seems to favor mechanisms for the fastest possible growth and the highest possible yield of biosynthesis. Therefore, the hydrolysis of ATP does not seem to be a physiologically important reaction. Reactions which produce ADP and P_i from ATP are doubtlessly

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

connected either with the utilization of the chemical energy of ATP for biosynthesis for transport, or with the biosynthesis of ATP itself, particularly at the expense of oxidative energy, but under *in vitro* conditions this ATP-synthesizing reaction is reversed, running down the energy scale.

We approached the study of membrane-bound ATPase activity with the aim of identifying an important step of oxidative phosphorylation as occurs in the mitochondrial system, where ATPase has been identified as the first "coupling factor"[1].

ATPase (EC 3.6.1.3) activity has previously been described in the membranes of several bacterial species[2-10], including *E. coli*[11,12]. The enzyme responsible for this activity has been solubilized and purified in some instances. Nevertheless, the physiological significance of these ATPases has not been unambiguously assessed and the properties expected for an ATPase involved in oxidative phosphorylation have not been clearly established in many cases. Recent reports of mutants of *E. coli* devoid of membrane ATPase activity and impaired in oxidative phosphorylation lend strong support to the involvement of this enzyme in the multienzymatic system of energy conversion[13-15].

Most membrane preparations previously used to study ATPase were obtained by osmotic lysis of lysozyme spheroplasts. Suspecting that properties of membrane-bound enzymes can undergo important changes due to procedures used in the preparation of membrane particles, we reexamined the properties of *E. coli* membrane ATPase in membrane preparations obtained by mechanical disruption of exponentially growing bacteria. We found that ATPase activity was essentially Mg^{2+} dependent, and responded to inhibitors of oxidative phosphorylation. This article describes some properties of the membrane-bound ATPase. Its solubilisation, with the corresponding changes in properties, and its reattachment to depleted membranes with restoration of the initial properties will be described in a subsequent paper.

MATERIAL AND METHODS

Bacteria

E. coli K12 strain 3300, which was used in all experiments, was grown in medium 63[16] supplemented with 4 g/l glycerol and 2 mg/l thiamine in aerated flasks at 37 °C for small preparations and in 15- or 50-l fermentors under forced aeration for larger batches. The medium used in the fermentors had the following composition: KH_2PO_4, 80 g; $K_2H\ PO_4$, 274.5 g; $(NH_4)_2SO_4$, 60 g; $MgSO_4$, 3 g; $FeSO_4$, 0.01 g; water, 15 l; glycerol, 8 g/l; thiamine, 2 mg/l.

Bacteria were harvested at an $A_{600\ nm}$ of 3 during exponential growth. They were sedimented in a Sharples continuous centrifuge, and washed with about 10 vol. of cold Tris–Mg^{2+} buffer containing 1 mM Tris–HCl (pH 7.6) and 1 mM $MgCl_2$, and resuspended in the same buffer in the cold.

Preparation of membranes

The bacterial suspension in cold Tris–Mg^{2+} buffer was disrupted in a Ribi cell fractionator by extrusion at a pressure of 30000 lb/inch^2 (approx. 2100 atmos), the temperature was kept below 15 °C at the extrusion nozzle.

Surviving bacteria and coarse debris were eliminated by centrifugation in the cold for 10 min at 5000 × *g*. The supernatant was centrifuged in a preparative ultra-

centrifuge (Beckman Spinco L 65) at 30000 × *g* for 20 min. The pellet contained particles enriched in cell wall material (lipopolysaccharides) and some membrane particles. The 30000 × *g* supernatant was centrifuged at 160000 × *g* for 180 min. The 160000 × *g* pellet, containing small membrane particles contaminated with some cell wall material and with ribosomes, was used in most experiments described below. Gel filtration on agarose Bio-Gel A-150 m (50–100 mesh) results in a preparation free of ribosomes and less contaminated with soluble enzymes. Properties of ATPase from such preparations are undistinguishable from those described below.

The 160000 × *g* pellet was washed once with cold Tris–Mg^{2+} buffer and stored (frozen) at −20 °C in small fractions in the same buffer at concentrations of 30–50 mg protein per ml.

ATPase assays

ATPase activity was assayed either by measurement of the inorganic phosphate liberated or by measurement of [^{14}C]ADP produced from [^{14}C]ATP. The incubation mixture routinely used contained 40 mM triethanolamine–HCl buffer, pH 7.5, (100–200 μg membrane preparation protein), 3.3 mM ATP sodium salt, pH 7.0, and 1.66 mM $MgCl_2$ (6.6 mM $MgCl_2$ in early experiments). The reaction was started after equilibration at 25 °C by addition of the ATP–Mg^{2+} solution. Inorganic phosphate was assayed after 4 min incubation in 0.5-ml samples according to the method of Martin and Doty[17].

Assays with an ATP-generating system contained besides the usual components 4 mM phosphoenolpyruvate and 30 μg pyruvate kinase (EC 2.7.1.40).

For measurement of [^{14}C]ADP, the same incubation mixture was used except that [8-^{14}C]ATP (C.E.A., France) was used at final specific activity of approximately 0.03 μCi/μmole. Samples of approximately 0.05 ml were mixed with 0.05 ml formic acid containing a mixture of non-radioactive ATP, ADP, AMP and adenosine. An aliquot of the sample was deposited on Whatman No. 3 paper and chromatographed for 10–12 h with the solvent isobutyric acid–1 M ammonia–1 mM EDTA (100:60:1.6, v/v/v). The four ultraviolet-absorbing spots were cut out and the radioactivity was counted by addition of 5 ml toluene-based scintillation mixture, in a liquid scintillation counter. ADP was determined as percent of the total counts recovered. No significant radioactivity could be detected outside the ultraviolet-absorbing spots.

Assay of ATP–ADP *exchange*

The radiochromatographic method was utilized for measuring ATP–ADP exchange except that [^{14}C]ADP was added to the incubation mixture instead of [^{14}C]ATP.

Adenylate kinase (EC 2.7.4.3) activity was variable in different membrane preparations but never exceeded 20 % of ATPase activity. [^{14}C]AMP produced was substracted from [^{14}C]ATP produced in order to calculate ATP–ADP exchange.

Activities are expressed as μmoles of product released per min per mg membrane protein. The results of exchange experiments were not extrapolated to initial velocity. In individual experiments the highest reactions proceeded as to 40 % toward isotopic equilibrium and therefore the velocities are slightly underestimated. Protein concentrations were measured according to the method of Lowry *et al.*[18].

[8-^{14}C]ADP was prepared by submitting [8-^{14}C]ATP to hydrolysis by a membrane preparation in the conditions described above except that a larger incubation volume was used and incubation was continued for 30 min at 25 °C. The whole incubation mixture was streaked on Whatman No. 3 paper, chromatographed with the solvent indicated and the ADP strip was cut out, eluted with 0.02 M HCl and lyophilized. The preparation contained approximately 1 % [^{14}C]ATP and 2 % [^{14}C]-AMP.

Chemicals

ATP, phosphoenolpyruvate were purchased from Boehringer; GTP, CTP, UTP, TTP, ADP, AMP, *N,N'*-dicyclohexylcarbodiimide (DCCD) were purchased from Calbiochem; pyruvate kinase was purchased from Sigma; [8-^{14}C]ATP was obtained from C.E.A., France; Dio 9 was purchased from Mycofarma Delft Holland; agarose was purchased from Bio Rad. All other chemicals were reagent grade.

RESULTS

A typical result of the distribution of ATPase activity during fractionation is summarized in Table I.

TABLE I

DISTRIBUTION OF ATPASE ACTIVITY

	Total protein (mg)	*Total activity (μmoles P_i/min)*	*Specific activity (μmoles P_i/min per mg protein)*
Unbroken bacteria	6000	<50	<0.01
Broken bacteria	6000	960	0.16
30000 × *g* pellet	1050	<50	<0.05
160000 × *g* pellet	2000	1000	0.50
160000 × *g* supernatant	3000	<50	<0.02

It was noticed in early experiments, that production of P_i was non linear *versus* time and *versus* protein concentration, unless an ATP-generating system was present (Figs 1 and 2). Fig. 1 shows that linearity did not extend beyond 6 min. This is classically interpreted as being due to product inhibition. If ADP was rephosphorylated by pyruvate kinase, the reaction remained linear for a longer time, and as also shown in Fig. 1 addition of ADP strongly inhibited the reaction, but again the reaction rate remained constant for 15 min or more. The reaction was strongly dependent on the Mg/ATP ratio. For Mg/ATP = 0.5 initial velocity was the same with or without an ATP-generating system. For Mg/ATP ratios higher than 0.5, the initial velocity was decreased in the absence of ATP-generating system and slightly increased in its presence.

Fig. 2 shows that the rate of reaction was proportional to the concentration of membrane, but at higher membrane concentrations without ATP-generating system the reaction deviated from linearity in less than 4 min.

The dependence on the Mg/ATP ratio is depicted in more detail on Fig. 3. In

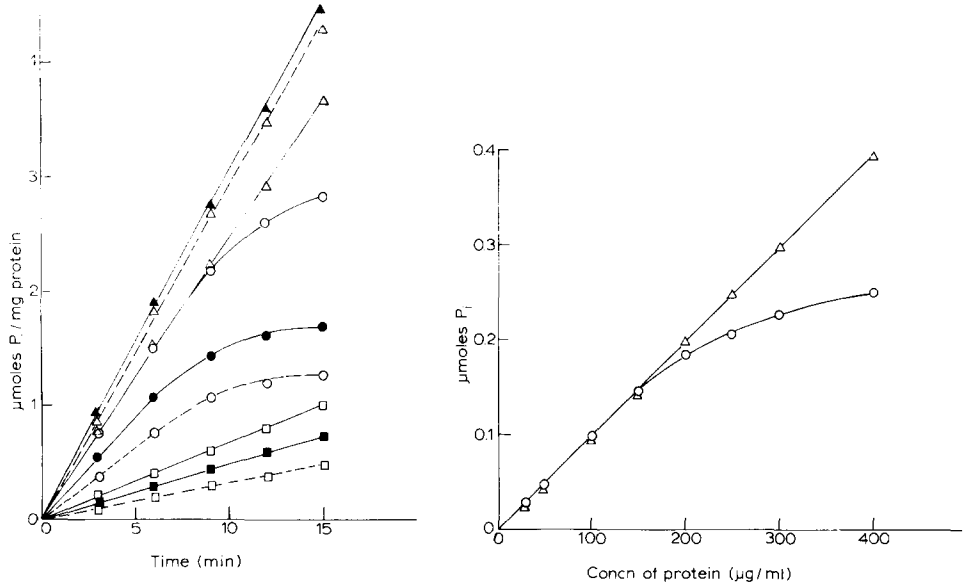


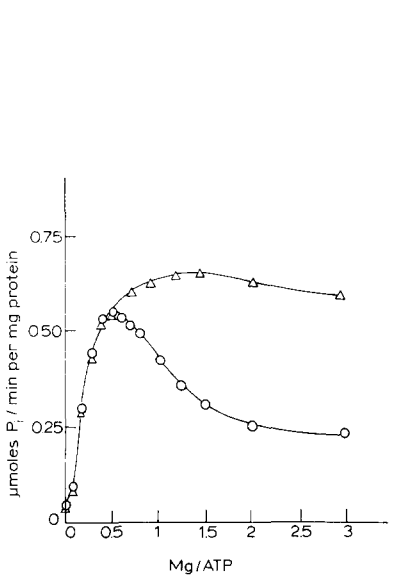
Fig. 1. ATPase activity *vs* incubation time at various Mg/ATP ratios. Experiments were carried out at pH 7.5 with 3.3 mM ATP. ○—○, Mg/ATP = 0.5; ●—●, Mg/ATP = 1; ○--○, Mg/ATP = 2. With ATP-generating system: △—△, Mg/ATP = 0.5; ▲—▲, Mg/ATP = 1; △--△, Mg/ATP = 2. With 2.3 mM ADP: □—□, Mg/ATP = 0.5; ■—■, Mg/ATP = 1; □--□, Mg/ATP = 2.

Fig. 2. ATPase activity *vs* the amount of membrane preparation. Experiments were carried out at pH 7.5 with Mg/ATP = 0.5. P_i was measured after 4 min incubation at 25 °C. ○—○, without ATP-generating system; △—△, with ATP-generating system.

the absence of added Mg^{2+} the activity of the system was very low. With Mg/ATP increasing up to 0.5, the reaction rate increased sharply and equally in both assays with or without an ATP-generating system, when Mg/ATP ratio was increased above 0.5, the activity observed without an ATP-generating system decreased to less than half of the maximal activity at Mg/ATP ratios of 2 or 3. In contrast, in the presence of phosphoenolpyruvate and pyruvate kinase the activity kept increasing when Mg^{2+} was increased until a flat maximum was reached at a Mg/ATP ratio of 1.5.

The divalent cation dependence of the membrane ATPase activity varied with pH. This is an important point, since in previous publications[11], the routine ATPase assay was carried out at pH 9 and it was concluded that ATP was Mg^{2+}, Ca^{2+} dependent. As shown on Fig. 4, ATPase activity without added divalent cations was negligible from pH 6 to pH 8 and increased sharply from pH 8 to pH 9. Ca^{2+} did not stimulate the activity between pH 6 and 7, an increasing Ca^{2+}-dependent activity was observed between pH 7 and 8 and basal activity was stimulated by Ca^{2+} in decreasing proportions between pH 8 and 9. At pH 9, activity was stimulated to approximately the same extent by Ca^{2+} and by Mg^{2+}. Maximum activity was observed at pH 7.5 with a Mg/ATP ratio of 0.5.

In *E. coli* an alkali cation-dependent ATPase activity has been described[19]. Addition of $Na^+ + K^+$ resulted in a 10–15 % increase above the Mg^{2+}-dependent



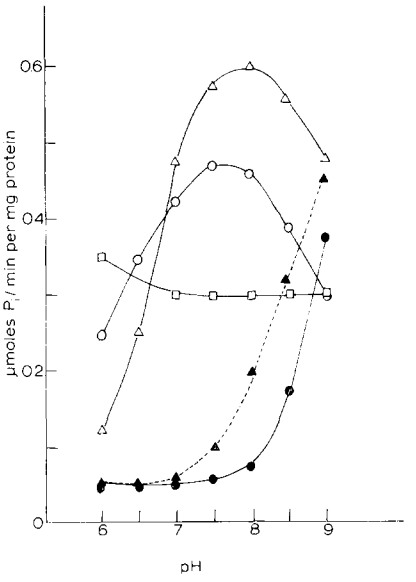


Fig. 3. Effect of Mg^{2+} on ATPase activity. ○—○, without ATP-generating system; △—△, with ATP-generating system. ATP concentration was 3.3 mM.

Fig. 4. ATPase activity *vs* pH in the presence of Mg^{2+} and Ca^{2+}. Experiments were carried out with 40 mM Tris–maleate buffer (pH 6–7.5), 40 mM triethanolamine–HCl buffer (pH 7.5–8.5), 40 mM Tris–HCl buffer (pH 9). ●—●, Mg/ATP = 0; △---△, Mg/ATP = 0.5; ○—○, Mg/ATP = 1; □—□, Mg/ATP = 2; ▲---▲, Ca/ATP = 0.5 and 1.

TABLE II

EFFECT OF ALKALI CATIONS ON ATPASE ACTIVITY

Experiments were carried out with 40 mM triethanolamine–HCl (pH 7.5), 3.3 mM ATP.

Divalent cations added	*Monovalent cations added*	*ATPase activity (µmoles P_i/min per mg protein)*
None	None	0.05
	Na^+ (1–100 mM)	0.05
	K^+ (1–100 mM)	0.05
	$Na^+ + K^+$ (1–100 mM)	0.05
Mg^{2+} (1.66 mM)	None	0.60
	Na^+ (100 mM)	0.45
	K^+ (100 mM)	0.60
	Na^+ (100 mM) + K^+ (100 mM)	0.45

activity. The effect of monovalent cations on the ATPase activity of our preparations is summarized in Table II. 100 mM Na^+ slightly inhibited the Mg^{2+}-dependent activity, whereas K^+ had no effect.

The ATPase activity *versus* ATP concentration exhibits Michaelis-type kinetics as shown on Fig. 5. The K_m for ATP was 0.5 mM and did not vary significantly with pH or with Mg^{2+} concentration within the limits of Mg/ATP 0.5–2.0. It has the same

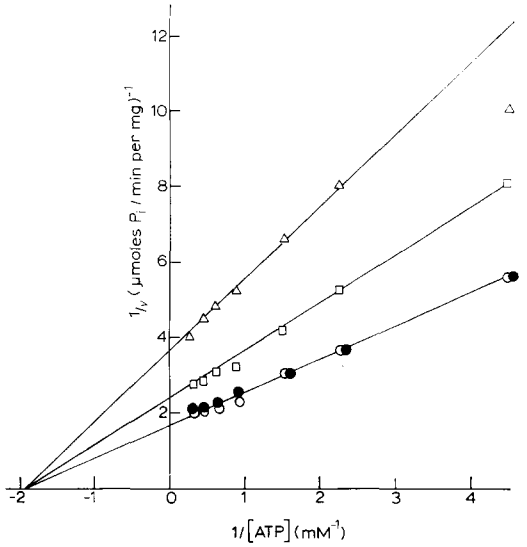


Fig. 5. Lineweaver–Burk plots of the ATPase activity *vs* ATP concentration. The reaction mixture contained: □—□, 40 mM Tris–HCl buffer (pH 9) without Mg^{2+}; ○—○, 40 mM triethanolamine–HCl buffer (pH 7.5), Mg/ATP = 0.5; ●—●, the same with ATP-generating system; △—△, 40 mM triethanolamine–HCl buffer (pH 7.5), Mg/ATP = 2.

value at pH 9 in the absence of added Mg^{2+} or in the presence of phosphoenolpyruvate and pyruvate kinase.

The inhibitory effect of ADP as a function of ATP concentration is represented in Fig. 6. In the presence of constant amounts of ADP, the ATP concentration giving half-maximal velocity increased with increasing ADP and the apparent V decreased. This complex type of inhibition was reminiscent of allosteric transitions with simultaneous effects on K_m and V. The Hill coefficient of the sigmoid curves was approximately 2. The search for allosteric effectors reversing the effect of ADP has been unsuccessful.

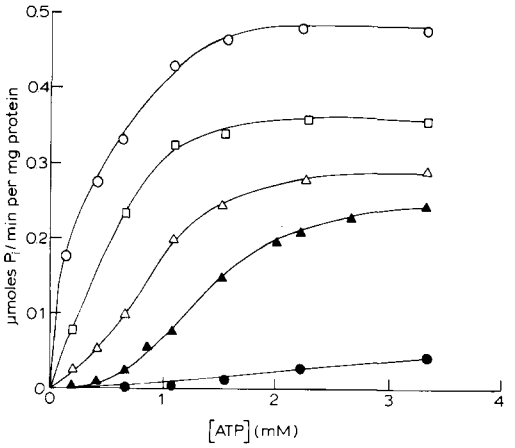
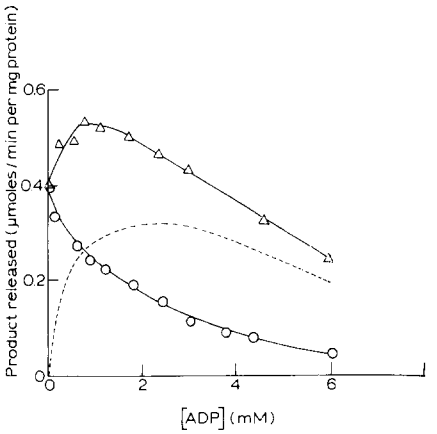


Fig. 6. ATPase activity *vs* ATP concentration in the presence of ADP. The reaction mixture contained 40 mM triethanolamine–HCl buffer (pH 7.5), Mg/ATP = 0.5; ○—○, ADP = 0; □—□, ADP = 0.4 mM; △—△, ADP = 1 mM; ▲—▲, ADP = 2 mM; ●—●, ADP = 5 mM.

When [^{14}C]ATP was used and [^{14}C]ADP was measured after chromatography in the presence of increasing concentrations of [^{12}C]ADP, the results shown on Fig. 7 were obtained. With no added ADP, the rates of release of P_i and of [^{14}C]ADP were equal. With increasing concentrations of ADP the release of P_i was strongly inhibited while the appearance of [^{14}C]ADP was strikingly stimulated. At 0.8 mM ADP the inhibition of P_i production was 35 % while [^{14}C]ADP produced was increased by 32 %. At higher ADP concentrations the rate of release of both products decreased, but the [^{14}C]ADP produced was always greater than the release of P_i, the difference being nearly constant and approximately two thirds of the initial velocity in the absence of ADP. This excess [^{14}C]ADP is obviously the result of an exchange reaction:

$$ATP^* + ADP \leftrightharpoons ADP^* + ATP$$

The velocity of this reaction can be measured without major interference of the hydrolytic reaction, by using [^{14}C]ADP and [^{12}C]ATP and measuring the production of [^{14}C]ATP. The results of two such experiments carried out with 3.3 mM ATP (saturating) and with 0.66 mM ATP (approximately half saturating) are represented in Fig. 8.



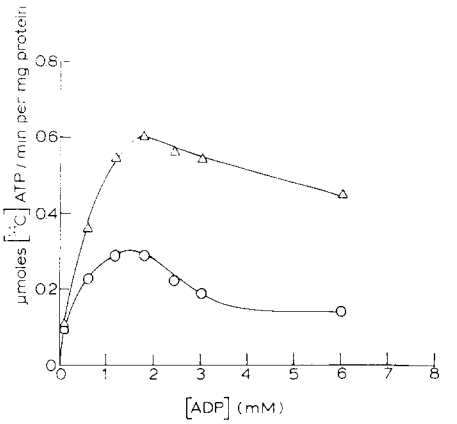


Fig. 7. Release of P_i and of [^{14}C]ADP from [^{14}C]ATP *vs* [^{12}C]ADP concentration. The reaction mixture contained 3.3 mM [^{14}C]ATP, 1.66 mM Mg^{2+}. ○—○, μmoles P_i/min per mg protein; △—△, μmoles [^{14}C]ADP/min per mg protein. – – – – –, difference between the two curves or velocity of ATP–ADP exchange.

Fig. 8. ATP–ADP exchange activity *vs* ADP concentration. △—△, ATP 3.3 mM, Mg^{2+} 6.6 mM; ○—○, ATP 0.66 mM, Mg^{2+} 1.33 mM.

Therefore, the inhibition of ATPase by ADP cannot be thought of as an allosteric inhibition as suggested by the results of Fig. 6, but rather as a channeling of the second half reaction of the following equation toward the upper branch.

$$ATP + E \leftrightharpoons ADP + P \sim E \begin{cases} \xrightarrow{ADP} ATP + E \\ \xrightarrow{H_2O} E + P_i \end{cases}$$

The first half reaction is stimulated by low concentrations of ADP and slightly inhibited by high concentrations.

The results of Fig. 6 can be reinterpreted as meaning that at low ATP concentrations the reaction was entirely channeled by ADP toward exchange, while at higher ATP concentrations the first half reaction became faster than exchange and the excess was overflowing toward hydrolysis. This explains the sigmoidal shape of the curve. That this interpretation is consistent is shown by the results depicted in Fig. 9, where production of P_i and of [^{14}C]ADP were measured simultaneously at various concentrations of [^{14}C]ATP.

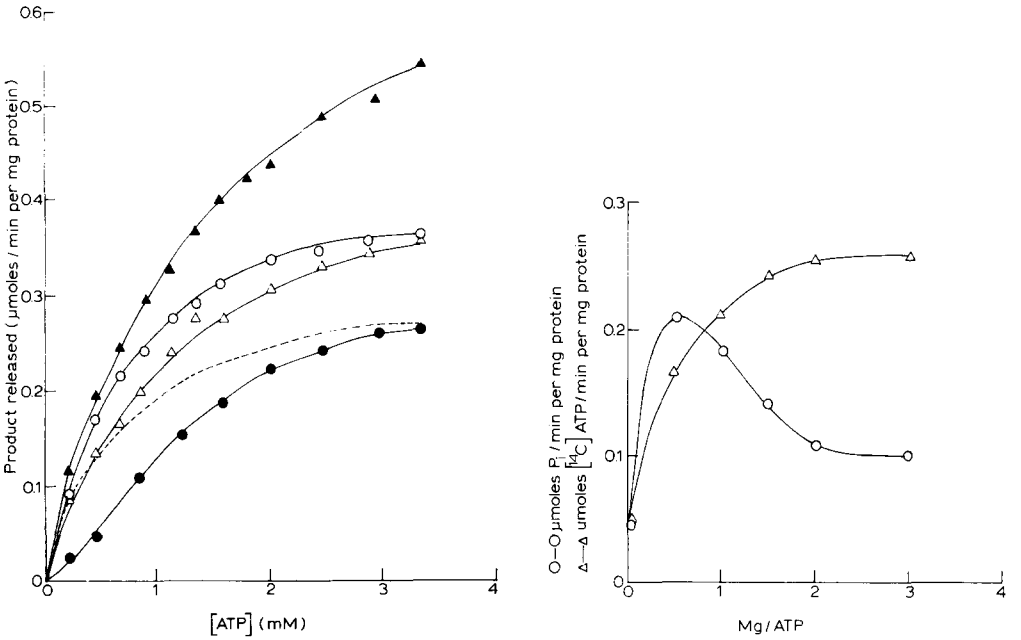


Fig. 9. ATPase activity *vs* ATP concentration in the presence of ADP. The reaction mixture contained 40 mM triethanolamine–HCl buffer (pH 7.5), Mg/ATP = 0.5. Without ADP: ○—○, μmoles P_i/min per mg protein; △—△, μmoles [^{14}C]ADP/min per mg protein. 0.8 mM ADP: ●—●, μmoles P_i/min per mg protein; ▲—▲, μmoles [^{14}C]ADP/min per mg protein; – – – –, difference between the last two curves ([^{14}C]ADP $-$ P_i).

Fig. 10. Effect of Mg^{2+} on the ATPase activity and on the ATP–ADP exchange activity. ○—○, the incubation mixture contained 40 mM triethanolamine–HCl (pH 7.5), 0.66 mM ATP; △—△, the incubation mixture contained 40 mM triethanolamine–HCl (pH 7.5), 0.66 mM ATP, 0.60 mM [^{14}C]ADP.

Similarly, the results of Fig. 3 can be reinterpreted as meaning that in the absence of ATP-generating system, the small amount of ADP released initially permitted an exchange reaction especially at high magnesium concentration, thus decreasing the rate of hydrolysis, while no such decrease was observed with the ATP-generating system, which prevents the possibility of ATP–ADP exchange. Accordingly, the results of the experiment of Fig. 10, show that the velocity of exchange was greatly increased when the Mg/ATP ratio increased from 0.5 to 2.0.

Specificity

Table III shows that the membrane preparation can release inorganic phosphate from a number of nucleoside triphosphates but had no hydrolytic action on nucleoside diphosphates. Moreover, the nucleoside triphosphates which can be hydrolyzed interfered with the rate of release of [^{14}C]ADP from [^{14}C]ATP, as shown on Table IV. Therefore, it seems likely that the same enzyme is involved in the hydrolysis of all the above-mentioned substrates.

TABLE III

SUBSTRATE SPECIFICITY OF NUCLEOTIDE PHOSPHATASE ACTIVITY

Experiments were carried out with 40 mM triethanolamine–HCl (pH 7.5), 1.66 mM Mg^{2+}, 3.33 mM of the indicated nucleotides.

Substrate	*μmoles P_i/min per mg protein*	*Activity relative to ATP*
ATP	0.39	100
GTP	0.31	80
CTP	0.13	32.5
UTP	0.07	17.9
TTP	0.04	10.5
ADP	0.00	0.00
UDP	0.00	0.00
CDP	0.00	0.00
GDP	0.00	0.00
AMP	0.00	0.00

TABLE IV

INHIBITION OF HYDROLYSIS OF [^{14}C]ATP BY VARIOUS NUCLEOTIDE TRIPHOSPHATES

Experiments were carried out with 40 mM triethanolamine–HCl buffer (pH 7.5), 1 mM ATP, 0.5 mM Mg^{2+}.

Additions		*μmoles [^{14}C]ADP/min per mg protein*	*% remaining activity*
	None	0.24	100
GTP	1 mM	0.15	62.5
	2 mM	0.10	41.5
	5 mM	0.03	12.5
CTP	1 mM	0.20	83.5
	2 mM	0.13	54.0
	5 mM	0.04	16.7
UTP	1 mM	0.16	66.5
	2 mM	0.10	41.5
	5 mM	0.04	16.7
TTP	1 mM	0.21	87.5
	2 mM	0.15	62.5
	5 mM	0.06	25.0

TABLE V

SUBSTRATE SPECIFICITY OF ADP–NTP EXCHANGE

Experiments were carried out at pH 7.5 with different nucleoside triphosphates (0.66 mM), 0.60 mM [8-^{14}C]ADP, 0.33 mM Mg^{2+}.

Phosphate donor substrate	*% radioactivity in ATP spot*	*% ATP − % AMP*	*μmoles [^{14}C]ATP/min per mg protein*	*Activity relative to ATP*
None	5.2	0	0	0
ATP	27.7	21.2	0.21	100
GTP	26.3	16.4	0.16	76
CTP	27.2	17.9	0.17	80
UTP	23.9	16.7	0.16	76
TTP	24.9	16.9	0.16	76

Table V also shows that various nucleoside triphosphates can donate a phosphate residue to [^{14}C]ADP to form [^{14}C]ATP, a reaction analogous to the exchange reaction described above.

Inhibitors

The effect of orthophosphate and of sodium azide on the membrane ATPase and on the ATP–ADP exchange is represented in Fig. 11. These two reagents, like ADP and high Mg^{2+}, inhibited the hydrolysis and stimulated the exchange.

The effect of a variety of inhibitors is listed in Table VI. Only inorganic pyrophosphate was found inhibitory for both activities.

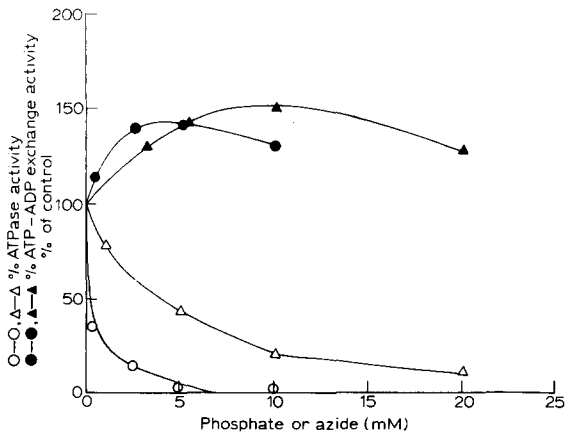


Fig. 11. Effect of orthophosphate and azide on the ATPase activity and on the ATP–ADP exchange activity. Incubation mixture contained 40 mM triethanolamine–HCl (pH 7.5), 3.3 mM [^{14}C]ATP and variable amounts of P_i (Δ—Δ). [^{14}C]ADP was measured. Incubation mixture contained 40 mM triethanolamine–HCl (pH 7.5), 3.3 mM [^{12}C]ATP, and variable amounts of azide (○—○), ADP was measured. Incubation mixture contained 40 mM triethanolamine–HCl (pH 7.5), 0.66 mM [^{12}C]ATP, 0.60 mM [^{14}C]ADP and variable amounts of P_i (▲—▲) or of azide (●—●). Production of [^{14}C]ATP was measured.

TABLE VI

EFFECT OF A VARIETY OF INHIBITORS

ATPase measurements were carried out at pH 7.5 with 3.3 mM ATP and 1.66 mM Mg^{2+}, exchange experiments were carried out at pH 7.5 with 0.6 mM [^{14}C]ADP, 0.66 mM ATP and 0.33 mM Mg^{2+}. n.d. = not determined.

Inhibitor	*Concentration*	*Activity (% of control)*	
		ATP hydrolysis	*ATP–ADP exchange*
Pyrophosphate	5 mM	41	30
	20 mM	0	0
DCCD	0.01 mM	25	100
	0.05 mM	10	100
Dio 9	50 μg/ml	55	100
	100 μg/ml	27	110
2,4-Dinitrophenol	0.50 mM	90	98
	1.00 mM	86	96
Arseniate	1 mM	100	100
	10 mM	100	100
Carbonylcyanide *m*-chlorophenylhydrazone	0.01 mM	100	n.d.
	0.05 mM	100	n.d.
N-Ethylmaleimide	1 mM	90	60
	20 mM	55	50

DISCUSSION

The ATPase activity described in this article exhibits important differences from the one described by Evans[11] in the same species. The differences concern pH dependence and activation by divalent cations. These differences are certainly due, for the most part, to differences between osmotically lysed lysozyme spheroplast ghosts and membranes from mechanically disrupted bacteria, and possibly to the difference in buffer composition during preparation. Since our preparations exhibit a more physiological pH optimum and the activity in the neutral pH range is strictly Mg^{2+} dependent we consider that its properties are more consistent with the assumed physiological role of ATPase than those described by Evans. Differences due to different strains cannot be ruled out presently.

The important question is to know whether: (a) The ATPase activity is due to a single enzyme, and if so, whether the enzyme is a part of the oxidative phosphorylation complex. (b) The bulk of the other nucleoside triphosphate phosphohydrolase activities are due to the same enzyme. (c) The nucleoside diphosphate kinase activities are due to the same enzyme.

The complexity of the membrane preparation and its limited purity preclude the possibility of deriving any strong support for the uniqueness of the enzyme(s). Only the membrane location of all the observed enzymes can be ascertained, since

preparations not contaminated with cell wall material, ribosomes and soluble proteins exhibit all the described activities.

Oxidative phosphorylation and the cation pump are the two main functions to which membrane ATPase activities are usually ascribed. K^+ transport in *E. coli* is known to be independent of the presence and of the transport of Na^+, and this diminishes the physiological significance of a possible ($Na^+ + K^+$)-dependent ATPase. ($Na^+ + K^+$)-dependent ATPase activities previously described as small increments of the basal Mg^{2+}-dependent activity remain, in the absence of specific inhibitors, unconvincing as separate and physiologically significant entities. In our preparations, we could not detect any significant activity dependent on alkali cations.

Support for the involvement of the ATPase here described in ATP synthesis comes from its inhibition by drugs which are well known inhibitors of oxidative phosphorylation. Although oligomycin is without action on the *E. coli* ATPase, as well as on all previously described bacterial ATPases, in contrast with its inhibitory effect on mitochondrial ATPase, DCCD, which has properties closely similar to oligomycin, is active. All efficient inhibitors tested exhibit a simple dose–effect curve.

Mutants of *E. coli* impaired in oxidative phosphorylation have been found not to contain membrane ATPase[13–15]. One of these mutants[15] was assayed with the present techniques, and the absence of ATPase could be confirmed.

It is likely that the described ATPase contributes to the hydrolysis of all the other triphosphates, but additional separate enzymes specific to one cannot be ruled out, since their competitive efficiency is not strictly proportional to their efficiency as substrates. The ATP–ADP exchange reaction might or might not be due to the ATPase. In favor of a single enzyme is the striking parallelism between the decrease of hydrolysis and the increase of exchange caused by ADP, Mg^{2+} and orthophosphate, which are supposed, according to the model presented, to deviate the reaction of the postulated phosphoenzyme from water to ADP as acceptor. The same deviation is observed under azide inhibition. In contrast, DCCD and Dio 9 simply inhibit hydrolysis and have no effect on exchange, and pyrophosphate inhibits both reactions. Other nucleoside triphosphates participate in hydrolytic and exchange reactions with roughly parallel efficiencies. Finally, both kinds of activities are removed from the membrane when ATPase is solubilized (unpublished results).

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